Microtiter Antiviral AIDS XTT Assay:

Cell Preparation:

CEM-SS cells (or other established human cell line used in these experiments) are passaged in T-150 flasks for use in the assay. On the day preceding the assay, the cells are split 1:2 to assure they are in an exponential growth phase at time of infection. On the day of assay the cells are washed twice with tissue culture medium and resuspended in fresh tissue culture medium. Total cell and viability counting is performed using a hemacytometer and trypan blue dye exclusion. Cell viability should be greater than 95% for the cells to be utilized in the assay.

The cells are pelleted and resuspended at 2.5×10^4 cells per ml in tissue culture medium. Cells are added to the drug-containing plates in a volume of 50 ul.

Virus Preparation:

A pretitered aliquot of virus is removed from the freezer (-80°C) and allowed to thaw slowly to room temperature in a biological safety cabinet. The virus is resuspended and diluted into tissue culture medium such that the amount of virus added to each well in a volume of 50 ul will be the amount determined to give complete cell killing at 6 days post-infection. In general, the virus pools produced with the IIIB isolate of HIV require the addition of 5 ul of virus per well. Pools of RF virus are five to ten fold more potent, requiring 0.5-1 ul per well. $TCID_{50}$ calculation by endpoint titration in CEM-SS cells indicate that the multiplicity of infection of these assays ranged from 0.005-2.5.

Plate format:

The format of the test plate has been standardized. Each plate contains cell control wells (cells only), virus control wells (cells plus virus), drug toxicity control wells (cells plus drug only), drug calorimetric control wells (drug only) as well as experimental wells (drug plus cells plus virus).

XTT staining of screening plates:

After 6 days of incubation at 37°C in a 5% $C0_2$ incubator, the test plates are analyzed by staining with the tetrazolium dye XTT. XTT-tetrazolium is metabolized by the mitochondrial enzymes of metabolically active cells to a soluble formazan product, allowing the rapid quantitative analysis of the inhibition of HIV-induced cell killing by anti-HIV test substances. On day 6 post-infection plates are removed from the incubator and observed. The use of round bottom microtiter plates allows rapid macroscopic analysis of the activity of a given test compound by the evaluation of pellet size. The results of the macroscopic observations are confirmed and enhanced by further microscopic analysis.

XTT solution is prepared daily as a stock of 1 mg/ml in PBS. Phenazine methosulfate (PMS) solution is prepared at 15 mg/ml in PBS and stored in the dark at -20°C. XTT/PMS stock is prepared immediately before use by diluting the PMS 1:100 into PBS and adding 40 ul per ml of XTT solution. Fifty microliters of XTT/PMS is added to each well of the plate and the plate is reincubated for 4 hours at 37°C. Adhesive plate sealers are used in place of the lids, the sealed plate is inverted several times to mix the soluble formazan product and the plate is read spectrophotometrically at 450 nm with a Molecular Devices Vmax plate reader. Using an in-house computer program %CPE Reduction, %Cell Viability, $IC_{25, 50 \& 95}$, $TC_{25, 50 \& 95}$ and other indices are calculated and the graphic results summary is displayed.

a. Reverse transcriptase activity assay:

A microtiter based reverse transcriptase (RT) reaction is utilized (Buckheit *et al* (1991) *AIDS Research and Human Retroviruses* 7:295-302). Tritiated thymidine triphosphate (NEN) (TTP) is resuspended in distilled H_20 at 5 Ci/ml. Poly rA and oligo dT are prepared as a stock solution which is kept at -20°C. The RT reaction buffer is prepared fresh on a daily basis and consists of 125 ul 1 M EGTA, 125 ul dH_20, 125 ul Triton X-100, 50 ul 1M Tris (pH 7.4), 50 ul 1 M DTT, and 40 ul 1 M $MgC1_2$. These three solutions are mixed together in a ratio of 1 part TTP, 2.5 parts poly rA:oligo dT, 2.5 parts reaction buffer and 4 parts distilled water. Ten microliters of this reaction mixture is placed in a round bottom microtiter plate and 15 ul of virus containing supernatant is added and mixed. The plate is incubated at 37°C and incubated for 60 minutes. Following reaction, the reaction volume is spotted onto filter mats, washed 6 times for 5 minutes each in a 5% sodium phosphate buffer, 2 times for 1 minute each in distilled water, 2 times for 1 minute each in 70% ethanol, and then dried. The dried filter mat is placed in a plastic sample bag, Betaplate scintillation fluid is added and the bag is heat sealed. Incorporated radioactivity is quantitated utilizing a Wallac Microbeta scintillation counter.

b. p24 ELISA:

ELISA kits are purchased from Coulter. The assay is performed according to the manufacturer's recommendations. Prior to ELISA analysis the reverse transcriptase activity assays (described above) are performed and the values used for incorporated radioactivity in the RT activity assay to determine the dilution of the samples required for the ELISA. Standard curves can be constructed so that the dilutions of virus to be used in the p24 ELISA can be accurately determined from the RT activity assay. Control curves are generated in each assay to accurately quantitate the amount of capsid protein in each sample. Data is obtained by spectrophotometric analysis at 450 nm using a Molecular Devices Vmax plate reader. P24 concentrations are calculated from the optical density values by use of the Molecular Devices software package Soft Max.

c. Infectious particles:

Infectious virus particles are quantitated utilizing the CEM-SS plaque assay as described by Nara, P.L. and Fischinger, P.J. (1988) Quantitative infectivity assay for HIV-1 and HIV-2 *Nature* 332:469-470). Flat bottom 96-well microtiter plates (Costar) are coated with 50 ul of poly-L-lysine (Sigma) at 50 ul/ml for 2 hours at 37°C. The wells are then washed with PBS and 2.5 x 10^5 CEM-SS cells are placed in the microtiter well where they became fixed to the bottom of the plate. Enough cells are added to form a monolayer of CEM-SS cells in each well. Virus containing supernatant is added from each well of the XTT plate, including virus and cell controls and each serial dilution of the test substance. The number of syncytia are quantitated in the flat-bottom 96-well microtiter plate with an Olympus CK2 inverted microscope at 4 days following infection. Each syncytium resulted from a single infectious HIV virion.